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SARCOIDOSIS: EXPRESSION OF CELL REGULATORY MARKERS AND THE INFLUENCE OF PATIENT PHENOTYPE ON BRONCHOALVEOLAR LAVAGE CELL DIFFERENTIAL COUNTS

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Sarcoidosis: Expression of cell regulatory markers and the influence of patient phenotype on bronchoalveolar lavage cell differential counts

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To my parents

ABSTRACT

Sarcoidosis is a systemic inflammatory disease that can affect almost any organ, but the respiratory system is affected in more than 90% of the cases. To elicit an immune response, an antigen(s) is processed by antigen-presenting cells (APCs), e.g. alveolar macrophages (AMs), and is presented in association with HLA-molecules to specific T cells, using their T cell receptor (TCR). In sarcoidosis, this interaction between the innate and adaptive immune system leads to an exaggerated immune response and formation of non-caseating granulomas in affected organs. The causative antigen remains elusive. To generate the sarcoid inflammatory process, the genetic background as well as exposure for antigens, endogenous or exogenous, is it of importance. Clinically, sarcoidosis patients can be divided into two major groups, i.e. patients with Löfgren's syndrome (LS) or with non-Löfgren's syndrome (non-LS). LS is a clinically distinct and well-defined phenotype that is characterized by an acute onset and is associated with specific HLA molecules, i.e. HLA-DRB1*03. In most of the LS patients, the disease resolves within two years. On the other hand, non-LS patients constitute a heterogeneous group and are prone to develop a chronic disease course.

Collecting cells from the deep lung compartment via bronchoalveolar lavage (BAL) enabled many researchers to explore immunological mechanisms in the alveolar space. In a healthy individual, BAL fluid (BALF) are mainly macrophages, some lymphocytes, and fewer neutrophils, and eosinophils; basophils and mast cells are rare. BALF from sarcoidosis patients contains an increased number of all these cell types, especially lymphocytes.

The first two studies (I, II) aimed to shed some light on the expression of cell regulatory markers in LS and non-LS patients. Macrophages are classically subdivided into two major subtypes, i.e. M1 – known as proinflammatory macrophages – and M2 – known as remodeling macrophages. We found in the first study (I) reduced gene expression of toll-like receptor 2 (TLR2: M1 associated marker) – mainly in LS patients – and increased expression of CCL18 (M2 associated marker) in AM of sarcoidosis patients. This finding could indicate a shift toward M2-like macrophages in sarcoidosis. The reduced TLR2 expression in LS patients might allow for a more effective immune response leading to resolution of granulomas. The CCL18 chemokine is known to act as T cell chemoattractant and can also induce collagen production in fibroblasts. Hence, the increased expression of CCL18 in AM of patients might attract T cells to the lung in the early stages of the disease and exhibit a profibrotic role in more advanced disease. The second study (II) explored the expression of specific transcription factors/nuclear receptors known to have regulatory roles in inflammatory diseases, i.e. the peroxisome proliferator-activated receptors (PPARs): PPARα, PPARβ/δ and PPARγ. Compared

to LS patients, PPARα expression was downregulated in BALF and blood $CD4^+$ and $CD8^+$ T cells in non-LS patients. Thus, CCL18 and PPARα could be used as biomarkers and might help in identifying patients at increased risk of developing more advanced lung disease.

The third study (III) aimed to explore the influence of patient phenotypes on BALF cell differential counts. We found that genetic variants associated with risk of LS and clustered in the extended MHC region were associated with the quantitative levels of BALF macrophages, lymphocytes, and neutrophils. Genetic variants associated with non-LS and located in the MHC II region associated with the quantitative levels of BALF macrophages only. In addition, these genetic variants exhibit regulatory effects on other genes in the lung, blood, T cells, B cells, macrophages and neutrophils.

The fourth study (IV) aimed to utilize data from a BALF registry of pulmonary sarcoidosis patients (LS and non-LS) to identify BALF cells that could predict disease severity (defined as advanced chest radiographs, reduced pulmonary function, or necessity for treatment) and/or chronicity (non-resolving course after two years). Compared with LS-resolving patients, LS-chronic patients exhibited higher BALF lymphocytes, neutrophils, and eosinophils. Additionally, in newly diagnosed LS patients, increased BALF neutrophils and basophils were more likely to associate with more severe disease; and increased BALF lymphocytes count was more likely to associate with a chronic disease course. In non-LS patients, increased BALF mast cells associated with a more severe and a chronic (non-resolving) disease, and increased BALF lymphocytes, neutrophils, eosinophils, and basophils associated with a more severe disease.

In summary, searching for biomarkers, we identified two possible markers for severe and/or chronic disease, i.e. PPARα and CCL18. In study III and IV, we showed that genetic variants associated with LS and non-LS can influence BALF cell counts and that increased BALF neutrophils, eosinophils, lymphocytes, basophils and notably mast cells have prognostic implication in newly diagnosed sarcoidosis patients.

LIST OF SCIENTIFIC PAPERS

I. Maria Wikén, Farah Idali, **MUNTASIR ABO AL HAYJA**, Johan Grunewald, Anders Eklund, and Jan Wahlström.

No evidence of altered alveolar macrophage polarization, but reduced expression of TLR2, in bronchoalveolar lavage cells in sarcoidosis. Respiratory Research, 2010, 11:121.

II. **MUNTASIR ABO AL HAYJA**, Anders Eklund, Johan Grunewald, and Jan Wahlström.

Reduced expression of peroxisome proliferator-activated receptor alpha in BAL and blood T cells of non-Löfgren's sarcoidosis patients. Journal of Inflammation, 2015, 12:28.

III. **MUNTASIR ABO AL HAYJA**, Susanna Kullberg, Anders Eklund, Leonid Padyukov, Johan Grunewald, and Natalia V. Rivera.

Influence of sarcoidosis risk genetic variants on the quantitative level of bronchoalveolar lavage fluid (BALF) cell populations. Submitted to Genes & Immunity.

IV. **MUNTASIR ABO AL HAYJA**, Jan Wahlström, Susanna Kullberg, Pernilla Darlington, Anders Eklund, and Johan Grunewald.

Bronchoalveolar lavage fluid cell subsets associate with the disease course in Löfgren's and non-Löfgren's sarcoidosis patients. Manuscript

CONTENTS

LIST OF ABBREVIATIONS

ACE	Angiotensin-converting enzyme
ADCY3	Adenylate cyclase 3
AM	Alveolar macrophage
ANXA11	Annexin A11
APCs	Antigen-presenting cells
AUC	Area under the ROC curve
BAL	Bronchoalveolar lavage
BALF	Bronchoalveolar lavage fluid
BCG	Bacilli Calmette-Guerin
BTNL2	Butyrophilin like 2
C10orf67	Chromosome 10 open reading frame 67
CCDC88B	Coiled-coil domain containing 88B
CCR	C-C motif chemokine receptor
CD	Cluster of differentiation
cDNA	complementary DNA
CI	Confidence interval
CLRs	C-type lectin receptors
COPD	Chronic obstructive pulmonary disease
CSMD1	CUB and Sushi multiple domains 1
CTLA-4	Cytotoxic T-Lymphocyte Associated Protein 4
CXCL	Chemokine (C-X-C motif) ligand
DAMPs	Damage-associated molecular patterns
DC	Dendritic cells
DLCO	Diffusion capacity for carbon monoxide
DNA	Deoxyribonucleic acid
$DR15^{pos}$	HLA-DRB1*15
$DR3^{pos}$	HLA-DRB1*03
FEV1	Forced expiratory volume in 1 second
FOXP3	Forkhead box P3
FVC	Forced vital capacity

GATA-3	GATA binding protein 3
HLA	Human leukocyte antigen
HRCT	High-resolution CT
ICAM-1	Intercellular Adhesion Molecule 1
IFN-γ	Interferon gamma
IL	Interleukin
iTreg	Induced T regulatory cells
LPS	Lipopolysaccharide
LR	Likelihood ratio
LRR	Leucine-rich repeat receptor
LS	Löfgren's syndrome
MCPH1	Microcephalin 1
MHC	Major histocompatibility complex class
mRNA	Messenger RNA
mTORC1	Mechanistic target of rapamycin complex
NF-kB	Nuclear factor kappa B
NK	Natural killer
NKT	Natural killer T
NOD	Nucleotide oligomerization
non-LS	Non Löfgren's syndrome
NR	Nuclear receptor
nTreg	Natural Treg cells
PAMPs	Pathogen-associated molecular pattern
PBMCs	Peripheral blood mononuclear cells
PD-1	Programmed Cell Death 1
PFTs	Pulmonary function tests
PGRs	Peptidoglycan recognition proteins
PLAC9	Placenta associated 9
PMA	Phorbol 12-myristate 13-acetate
PPARs	Peroxisome proliferator-activated receptors
PPD	Purified protein derivative

PRRs	Pattern recognition receptors
PSMB2	Proteasome Subunit, Beta Type, 2
P-value	Probability value
RIG-I	Retinoic acid-inducible gene I
RNA	Ribonucleic acid
ROC curve	Receiver operating characteristic curve
RORγt	Retinoic acid receptor-related-orphan-receptor-gamma t
RT-PCR	Real-time polymerase chain reaction
RXR	Retinoid X receptor
SAA	Serum amyloid A
STAT	Signal Transducer And Activator Of Transcription
T-bet	T-Box Transcription Factor
TCR	T cell receptor
TGF-β	Transforming Growth Factor Beta
T_H	T helper cells
TLC	Total lung capacity
TLRs	Toll-like receptors
TNF-α	Tumor necrosis factor alpha
VCAM-1	Vascular cell adhesion protein 1
WASOG	World Association of Sarcoidosis and Other Granulomatous Disorders

1 THE LUNG

The primary function of lung parenchyma is gas exchange. The lungs are continuously exposed to multiple environmental agents and diverse microorganisms. Therefore, the lungs have a critical role in immunity. The respiratory system consists of airways and lung tissue. The left lung contains two lobes: an upper and lower lobe. The right lung contains three lobes: an upper, middle and lower lobe. Each lung has ten bronchopulmonary segments. The airways consist of the trachea (windpipe) which divides into two main bronchi, i.e. the left and the right bronchus. The two main bronchi continue to branch into lobar, segmental, and smaller airways which continue to divide until they reach 1mm in diameter. Bronchioles have no cartilage and include the terminal and respiratory bronchioles (0,5 mm diameter) which are last airways before the small airway sac starts (alveolus). The lungs contain approximately 300 million alveoli with a surface area of about 70 m^2.

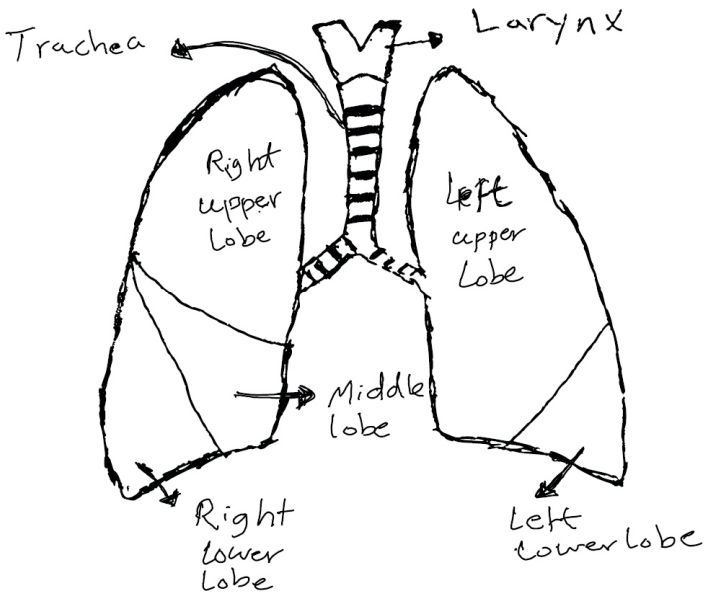

Figure 1. *Gross anatomy of the lung simplified.*

2 THE IMMUNE SYSTEM

Historically, innate and adaptive immune responses have been treated as separate, and the latter being considered more advanced. It is now clear this is not the case. Innate immune responses provide the essential early controls and deconditioning needed for an efficient adaptive immune response. Innate responses occur within minutes or hours of infection, whereas initiation of an effective adaptive immune response may take days.

2.1 Innate Immunity

The innate immune system is an evolutionary ancient defense system that evolved to protect the host against microbial pathogens. The innate immune system relies upon recognition of conserved molecular features of microbial pathogens or damaged cells [1]. The innate immune responses are characterized by the rapidity of action. Most cells of the system are derived from myeloid precursors from the bone marrow. These include monocytes and their derivatives-macrophages and dendritic cells-, granulocytes (neutrophils, basophils, and eosinophils), and mast cells. Natural killer (NK) and natural killer T (NKT) cells are derived from precursor cells of the lymphoid lineage. Innate immune dysfunction contributes to common conditions of multifactorial etiology such as sarcoidosis [2, 3].

Pattern recognition receptors (PRRs)

PRRs play a crucial role in the innate immune response by recognizing danger signals that arrive either from microorganisms [pathogen-associated molecular patterns (PAMPs)] or damaged cells [damage-associated molecular patterns (DAMPs)].

PRRs can be subdivided by their function and location into three major subtypes: 1 – membrane-bound PRRs: TLRs and C-type lectin receptors (CLRs), 2 – cytoplasmic (intracellular) PRRs: Nucleotide oligomerization (NOD) like receptors and retinoic acid-inducible gene I (RIG-I) like receptors, and 3 – secreted PRRs that bind directly to invading micro-organisms [e.g. peptidoglycan recognition proteins (PGRs) and the leucine-rich repeat receptor (LRR)].

Human have ten TLRs that recognize a broad spectrum of pathogens. TLR2 recognizes microbial components, which include lipoteichoic acid of gram-positive bacteria and lipoproteins of gram-negative bacteria. TLR4 signals the presence of bacterial lipopolysaccharide (LPS) of gram-negative bacteria. Another Toll-like receptor, TLR9 recognizes bacterial and viral unmethylated CpG DNA within endosomes [4].

Upon detection of a pathogen, the intracellular domain of these receptors initiates the activation of the transcription factor NFκB that stimulates the production of co-stimulatory (CD80 and CD86) molecules, cytokines (e.g., IL-1, IL-6, IL-12, and TNF-α), and chemokines (e.g. CXCL8) involved in anti-microbial defense and inflammation. Expression of the co-stimulatory molecules is necessary for the activation of naive $CD4^+$ T cells and thus initiating the activation of the adaptive immunity [1, 5, 6].

Macrophages

In 1892, Ilya Mechnikov (1845–1916) discovered specific white blood cells that were able to engulf and digest bacteria by a process that he named phagocytosis. He called the cells according to their size: macrophages and microphages (neutrophils) [7]. Nicolas Tchistovitch (1860–1926) was the first to describe alveolar macrophages (AMs) that are capable of phagocytosis [8]. Circulating blood monocytes were thought to be the sole origins of tissue macrophages. Now we know that most of the alveolar macrophages originate, during embryonic life, from progenitor cells (from the yolk sac and fetal liver) that migrate to the lung and mature locally before or shortly after birth [9, 10]. These alveolar macrophages are long-living cells and have the capability of self-renewal [11]. Several types of macrophages reside in the lung: bronchial macrophages, interstitial macrophages, intravascular macrophages (located on the inner side of capillaries), and AMs. Interstitial macrophages are found in the interstitium where they interact with dendritic cells and interstitial lymphocytes [10, 12]. Under normal homeostasis, AMs activation is tightly controlled through several cell-cell interactions and soluble mediators to limit uncontrolled inflammatory responses [13]. Under specific conditions such as infection, inflammatory diseases, pollution and smoking, blood monocytes are recruited into lung tissue and differentiate into monocyte-derived AMs [11].

Macrophages are critical sensors for the immune system, and therefore they are equipped with many receptors. Macrophages can sense danger signals through receptors that are called pattern recognition receptors (PRRs) [5].

AMs can activate antigen-experienced T cells to a similar extent as dendritic cells (DCs), but not naïve T cells [14]. Macrophages also have opsonic receptors that favor phagocytosis such as the complement receptors or immunoglobulin receptors. Macrophages are equipped with receptors for various chemokines, inflammatory cytokines (e.g. IL-1, TNF-α, IFN-γ, IL-17) and for the anti-inflammatory cytokines (e.g. IL-10 and TGF-β). Macrophages have adhesion receptors (e.g. ICAM1, VCAM1, or the β-integrins). Macrophages can be modulated by negative signals (e.g. acetylcholine, lipoxin) [15, 16].

Macrophages can undergo specific differentiation into M1 (classically activated) or M2 (alternatively activated) phenotype in response to DAMPs, PAMPs, cytokines, and other mediators released in the local tissue microenvironment [17, 18]. LPS and IFN-γ prompt the polarization of macrophages towards the M1 phenotype. M1 macrophages secrete large amounts of cytokines such as IL-1β, TNF-α, IL-12, IL-18, and IL-23. Thus, M1-macrophages promote antigen-specific T_H1 and T_H17 cell inflammatory responses. Phenotypically, M1 macrophages express high levels of MHC II, and co-stimulatory molecules CD80 and CD86. M2 macrophages are subdivided into M2a, M2b, M2c, and M2d according to their gene expression profile. M2a macrophage is elicited by fungal and helminth infections, IL-4, or IL-13. M2b macrophage is elicited by IL-1 receptor ligands, and immune complexes and LPS. TGF-β, IL-10, and glucocorticoids elicit M2c macrophage [19]. IL-6 and adenosine elicit M2d macrophages. In healthy individuals, AMs do not precisely fit into either a rigid M1 or M2 classification [13, 20].

Neutrophils

Gabriel Andral (1797–1876) and William Addison (1802–1881) were the first to describe leukocytes 1843 [21]. Paul Ehrlich (1854–1915) was the first to describe neutrophils, basophils, eosinophils and mast cells in 1879 [7, 21-23]. Besides their established role as a first-line defense in infectious disease, neutrophils play an essential role in chronic inflammation, adaptive immune responses, and autoimmunity. Neutrophils can produce IFNγ and act as antigen-presenting cells (APCs) [24-28].

Basophils

Basophils are derived from hematopoietic stem cells. They have a short lifespan (several days) and make up <1% of the blood leukocytes [22]. It is now well established that basophils not only function as pro-inflammatory cells but also modulate the immune response in many ways. Basophils can be activated by IgE crosslinking to undergo rapid degranulation of granules [4]. Basophils express TLRs and can be activated by PAMPs [22, 29]. Activated basophils can secrete IL-4 and promote T_H2 differentiation [30, 31]. Upon activation, basophils express MHC II, and co-stimulatory molecules and can activate naive $CD4^+$ T cells by acting as APCs [31, 32]. Basophils can augment T_H17 and T_H17/T_H1 effector responses [33]. Basophils can enhance the suppressive activity of $FOXP3^+$ regulatory T cells [34].

Eosinophils

Eosinophils are derived from hematopoietic stem cells. Eosinophils can express MHC II and co-stimulatory molecules, process antigens and can activate and interact with T cells to proliferate and produce cytokines in an antigen-specific manner [35]. Eosinophils express several families of pattern-recognition receptors (PRRs),

e.g. eosinophils express TLRs, although at lower levels than by neutrophils and macrophages [35]. Eosinophils express multiple T_H1, T_H2, and immunoregulatory cytokines, including IL-4, IL-13, IL-6, IL-10, IL-12, IFN-γ, and TNF-α, that are secreted rapidly and differentially in response to specific stimuli to promote either T_H2 or T_H1 cell responses [35]. Furthermore, a large quantity of IFN-γ was secreted in response to T_H1, T_H2, and inflammatory stimuli [35-37].

Mast cells

Mast cells are long-lived tissue-resident cells [38]. Mast cells derive from hematopoietic stem cell but undergo terminal differentiation in tissue [39]. The lungs do not have numerous mast cell progenitors in a healthy physiological state. Upon antigen-induced inflammation, mast cell progenitors are recruited to the respiratory endothelium. When mature mast cells are activated, more mast cell progenitors are recruited to the site of inflammation [40]. In addition to their primary function in IgE-mediated allergic or anaphylactic responses, mast cells can mediate several modulatory effects. Mast cells produce cytokines than can drive $CD4^+$ T cells and Treg cells to become IL-17 producing cells [41, 42]. Furthermore, mast cells can modulate dendritic cell maturation and function to promote T_H1 and T_H17 responses [43]. Mast cells can recognize PAMPs via TLRs [39]. The expression of MHC class II and co-stimulatory molecules can be induced on human mast cells and these mast cells can act as antigen-presenting cells [44, 45]. Mast cell proteases can have anti-inflammatory activities, mainly through the degradation of pro-inflammatory cytokines and chemokines.

Dendritic cells: the link between innate and adaptive immunity

During pathogen invasion, DCs can recognize PAMPs through PRRs such as TLRs and become activated. Upon activation, DCs enhance their expression of class I and II MHC and co-stimulatory molecules. DCs become mature DCs and can then migrate to the draining lymph nodes. Dendritic cells are the only cells capable of stimulating naive $CD4^+$ or $CD8^+$ T lymphocytes through presentation of class I or II MHC-restricted antigen (peptide) to naive $CD8^+$ or $CD4^+$ T cells respectively. DCs play a crucial role in initiating tolerance, and polarized T_H1, T_H2 and T_H17 differentiation [46].

Trained innate immunity

Recently, the historical view that immunological memory is only a feature of the adaptive immunity has been challenged. Monocytes and macrophages that are exposed to microbial components (e.g., beta-glucan) or vaccination (e.g., with bacilli Calmette-Guerin, BCG) undergo epigenetic modifications that lead to increased cytokine production upon re-stimulation. NK cells showed features of trained immunity after BCG vaccination and can produce increased levels

of proinflammatory cytokines (IL-1β, IL-6 and TNF-α) [47]. Excessive trained immunity was proposed in different disease, e.g. inflammatory and autoimmune disorders, allergies, cardiovascular diseases, specific infection and cancer [48]. Compared with blood monocytes from healthy individuals, blood monocytes from sarcoidosis patients express more TLR2 and TLR4 at baseline, and under certain conditions produce more inflammatory cytokines (TNF-α, IL-1β) [49]. Moreover, RNA-sequencing of blood monocytes of sarcoidosis patients revealed upregulation of genes involved in phagocytosis and lysosomal pathway. These results indicate that trained immunity could be a feature in sarcoidosis [50].

Human leucocyte antigen (HLA)

In human, the major histocompatibility complex (MHC) region is located on the short arm of chromosome 6. In humans, this region is called human leukocyte antigen (HLA). Genes in this complex are subdivided into the extended class I, classical class I, classical class III, classical class II and extended class II regions [51]. Humans have three main MHC class I genes, i.e. HLA-A, HLA-B, and HLA-C. There are six main MHC class II genes in humans: HLA-DPA1, HLA-DPB1, HLA-DQA1, HLA-DQB1, HLA-DRA, and HLA-DRB1. The MHC class III region encodes various molecules important in the inflammatory response, e.g. complement components C2, C4, and factor B, and tumor necrosis factor-alpha (TNF-α). The function of the HLA class I and II molecules is to bind peptides and present them to the T-cell receptor (TCR) on T cells. HLA genes are closely linked, and the entire MHC is inherited as an HLA haplotype. The HLA region is associated with diseases (mainly autoimmune and infectious diseases) more than any other region of the genome [4, 52].

The class II region contains the classical class II alpha (a) and beta (b) chain genes, HLA-DP, -DQ, and -DR that are expressed on the surface of APCs to present peptides to T-helper cells. In the nomenclature of genes in class II, HLA indicates the gene region; the first letter indicates the class (D); the second indicates the family (P, Q, or R) and the third letter designates the chain A (α) or B (β) followed by an asterisk that acts as a separator, followed by the number of the allele family and followed by the number of an allele variant. For example, HLA-DRB1*0301 means the allele variant 01 of the allele family 03 that encodes the β chain of class II molecule of the DR family [52-54].

2.2 Adaptive Immunity

The adaptive immune response has two main characteristics: it can respond flexibly to new, previously unencountered antigens (antigenic specificity) and its potential to respond to previously encountered antigen (immunological memory) [2, 55-57].

T-cells

T cells are predominantly produced in the thymus and that is the cause for naming them T cells. T cells are subdivided into two lineages, $CD4^+$ T cells (T helper, T_H cells), and $CD8^+$ T cells (cytotoxic T cells).

TCRs are made up of pairs of chains: either α and β chains or γ and δ chains. However, γδ T cells are much less common than αβ T cells. T cell receptor α and β chains consist of variable (V) region and a constant (C) region. The TCRβ locus (chromosome 7) consists of variable (V), diversity (D), and joining (J) gene segments, and a constant (C) gene. The organization of TCRα locus (chromosome 14) is similar but with the absence of the D segment.

TCR is associated with CD3 complex that transmits activation signals into the cell when the T-cell receptor binds the peptide (antigen)-MHC complex [56].

Naïve $CD4^+$ T cells

Human naive CD4+ T cells have an expected lifespan of 6–9 years. Naive $CD4^+$T cells circulate between lymphoid organs (e.g. lymph nodes and spleen) and blood. In humans, naive $CD4^+$ T cells express CD45RA and lack the expression of the memory-associated marker CD45RO [4]. Mature dendritic cells migrate to secondary lymphoid organs (such as regional lymph nodes) and interact with antigen-specific naïve $CD4^+$ T cells. Three kinds of signals are required for activation and polarization of these naive $CD4^+$ T cell. The first signal is the interaction of peptide-MHC II complex on dendritic cells with TCR on naive $CD4^+$ T cells. The second signal is the interaction of co-stimulatory molecules such as CD80 or CD86 on dendritic cells with the CD28 receptor on naive $CD4^+$ T cells. The third signal is the cytokines provided by the local environment that has a considerable impact on the differentiation of naive $CD4^+$ T cells into the effector subtypes, including T_H1, T_H2, T_H17, and induced T regulatory cells (iTreg) [58].

T_H1

IFN-γ and IL-12 are the central cytokines that promote the development of T_H1 cells. IFN-γ induces the activation of the transcription factor STAT1, which induces the expression of the transcription factor T-bet. T-bet switches on the genes for IFN-γ and IL-12 receptor. IL-12 leads to the activation of the transcription factor STAT4, which leads to the expansion and differentiation of T_H1 cells [59, 60].

T_H2

IL-4 promotes the development of T_H2 from naïve $CD4^+$ T cells. T_H2 cells produce IL-4, IL-5, and IL-13 and express the transcription factor GATA-3. Epigenetic regulation of GATA-3 is vital to sustaining the T_H2 identity [59-61].

T_H17 T cells

T_H17 cell subset was first identified in 2005 in the mouse as $CD4^+$ T cells that produce IL-17 following in vitro activation. T_H17 cells develop from antigen-activated naive $CD4^+$ T cells in the presence of the cytokines IL-6, IL-21, and TGF-β. They are characterized by expression of the transcription factor RORγt and produce the cytokines IL-17A, IL17F and IL-22. The cytokines produced by T_H17 acts on endothelial cells to induce the production of chemokines that recruit neutrophils to the site of infection. IL-23 is essential in amplifying the generation of T_H17 cells and sustaining their survival. It is important to recall that cells of the innate and adaptive immune system other than T_H17 cells, produce IL17 in vitro and in vivo. T_H17 cells are usually identified (utilizing flow cytometry) by intracellular staining of IL-17, often following massive stimulation (e.g. with PMA/ionomycin). At this stage, these cells show important plasticity and can differentiate into effector or regulatory T cell subset. In tissue, where the local secretion of IL-17 occurs, the cells have a different morphology and appear to produce a single cytokine at a time. Both lymphocyte activation and cell contact with mesenchymal cells are critical for a high level of IL-17-production. Therefore, it is important to distinguish between intracellular staining and actual secretion of IL-17 [62].

Regulatory T cells (Treg cells)

Tregs are T cells which have a role in suppressing other cells in the immune system. Tregs produced in the thymus are termed natural Treg cells (nTreg). Treg induced by differentiation of naive T cells outside the thymus, i.e., the periphery, or in cell culture are called 'induced' or 'adaptive' Treg cells (iTreg). Tregs are $CD4^+$, $CD25^+$ (the alpha chain of the IL-2 receptor), express the transcription factor FOXP3 and also express low levels of CD127 (the alpha chain of the IL-7 receptor). However, activated $CD4^+$ T cells show expression of CD25, and 'effector' $CD4^+$ T cells express FOXP3 upon activation. Accordingly, expression of CD25 and FOXP3 cannot be used with confidence to quantify Treg cells. Hence, an in vitro assay that measures the ability of Treg cells to suppress $CD4^+$ T cell proliferation might be used to test for Treg function. Tregs control inflammation and immune responses by diverse mechanisms of suppression: (1) secretion of the immunosuppressive cytokines IL-10, TG-β, and IL-35; (2) IL-2 consumption; (3) cytolysis of effector cell by producing granzyme and perforin; and (4) direct contact with dendritic cells through inhibitory co-receptors such as CTLA-4 [62, 63].

T_H17/Treg balance

TGF-β can induce expression of FoxP3 and RORγt in TCR-stimulated naïve CD4 + T cells. In inflammatory conditions, FoxP3 expression is reduced, and RORγt expression is up-regulated that promotes T_H17 differentiation. In the absence

of inflammation, TGF-β promotes Treg differentiation; this is due to a FoxP3-mediated inhibition of RORγt. The developmental pathways of T_H17 and Treg cells are reciprocally regulated and can affect the outcome of immune responses, especially in autoimmune and inflammatory diseases. [62, 64].

$CD8^+$ T cells

$CD8^+$ T cells are crucial for immune defense against intracellular pathogens, including viruses and bacteria, and for tumor surveillance. $CD8^+$ T cells recognize peptides presented by MHC Class I molecules, found on all nucleated cells. Activated $CD8^+$ T cells secrete cytokines such as TNF-α and IFN-γ, which have anti-tumor, anti-viral and anti-microbial effects. $CD8^+$ T cells kill their targets by inducing them to undergo apoptosis.

3 PEROXISOME PROLIFERATOR-ACTIVATED RECEPTORS (PPARS)

The peroxisome proliferator-activated receptors (PPARs) are ligand-activated transcription factors that have a crucial role in various cellular processes, including regulation of immune responses and inflammation [65]. PPARs are members of the nuclear receptor (NR) superfamily. There are three subtypes of PPARs, i.e. PPARα, PPARβ/δ, and PPARγ. The PPARs are expressed in different cells, including macrophages and T lymphocytes [66, 67]. PPARs can be activated by natural (e.g. fatty acids, prostaglandins, leukotriene) and several synthetic ligands (e.g. fibrates and glitazones). After a ligand binding, PPAR translocates to the nucleus to heterodimerize with the retinoid X receptors (RXRs) thereby activating the expression of different genes (transactivation by direct binding to specific DNA response elements). Also, PPARs can downregulate the expression of pro-inflammatory genes by interfering with other transcription factors, e.g. NF-kB, STAT-1, and AP-1, but without direct binding to the DNA [66]. Hence, activating PPARα, PPARβ/δ, and PPARγ by ligands can inhibit T_H1 pro-inflammatory cytokine production by activated T cells . PPARα, and PPARγ exhibit immunomodulatory and anti-inflammatory effects in various inflammatory diseases, including pulmonary diseases such as COPD and asthma [68-72].

4 SARCOIDOSIS

Sarcoidosis is a granulomatous disease of unknown etiology that can affect any organ but mainly affect the lung in >90% of the cases. Sarcoidosis is a multifactorial disease where interaction between environmental and genetic factors contribute to the pathogenesis of the disease.

4.1 Historical overview

Sir Jonathan Hutchinson (English surgeon, ophthalmologist, dermatologist, and pathologist; 1828-1069) was the first to describe a (sarcoid) skin plaque in London 1869. In 1889, Ernest Besnier (French dermatologist; 1831-1909) described skin lesions associated with sarcoidosis as “lupus pernio”. In 1899, Caeser Boeck (Norwegian dermatologist; 1845-1917) used the term “multiple benign sarkoid of the skin”. Boeck used the term “sarkoid” because the lesions resembled sarcoma. Boeck was the first to describe the granulomatous histology of sarcoidosis and the involvement of multiple organs (e.g. the lungs, lymph nodes, and spleen). In 1909, Christian Friedrick Heerfordt (Danish ophthalmologist: 1871-1953) described a syndrome characterized by skin lesion, enlargement of parotid and submaxillary salivary glands, uveitis, and paresis of the cranial nerves and termed it “febris uveo-parotidea subchronica”. In 1914, Jörgen Nilsen Schauman (Swedish dermatologist; 1879-1953) described the systemic nature of the disease as “lymphogranuloma benigna”. Finally, Sven Löfgren (Swedish physician; 1910-1978) described 1953 a syndrome consisting of fever, bilateral hilar lymphadenopathy, polyarthritis, and erythema nodosum that is now known as Löfgren’s syndrome [73, 74].

4.2 Epidemiology of sarcoidosis

The incidence and prevalence of sarcoidosis vary widely. The highest prevalence is reported in northern Europeans and African Americans. The prevalence and incidence of sarcoidosis in Sweden are 160 and 11,5 per 100 000 per year respectively [75]. In the USA, the prevalence is 141 and 50 per 100 000 in African Americans and white people, respectively [76]. The incidence in Japan is much lower, 1.01 per 100 000 [76].

In Sweden, individuals with sarcoidosis had a higher mortality rate (11 per 1000 person-years) as compared to matched individuals from the general population (6.7 per 1000 person-years) [77].

Pulmonary fibrosis is detected in 4.7–15% of patients at presentation, with a subsequent higher mortality rate as compared with the general population. Mortality is related to respiratory causes in 75% of cases [78]. Less than 5% of patients die from sarcoidosis [79].

4.3 Etiology

The etiology of sarcoidosis remains speculative. Several lines of evidence point towards that sarcoidosis is an exaggerated immune response to persistent antigens from inorganic and/or organic agents or bacterial antigens derived from incompletely degraded bacteria such as *Mycobacteria* and *Cutibacterium acnes* (previously known as *Propionibacterium acnes*). Vimentin is an endogenous antigen that might be involved in the pathogenesis of sarcoidosis. The unknown antigen probably varies according to the geographic distribution and occupational exposure.

It is likely that no single antigen is responsible for sarcoidosis but rather that various antigens could cause the disease. These antigens could be exogenous or endogenous, and this might explain the diversity in disease localization within the body [80-82].

4.4 Candidate gene association studies and Genome-wide association studies

A various number of non-HLA genes have also been associated with risk of disease or phenotype. Examples include the association with the following genes: TGF-β, IL-23 receptor, TLRs, CCR2, and CCR5 [81, 83]**.**

Genome-wide association studies revealed several loci that are associated with increased risk of sarcoidosis: *C10orf67*, *ANXA11*, *BTNL2*, *CCDC88B*, and others [84-90].

Rivera *et al*, performed fine mapping analysis -using Immunochip- and identified 727 LS-associated variants in the extended MHC region and two loci in *ADCY3* (2p23.3) and between *CSMD1* and *MCPH1* (8p23.1 - 8p23.2) outside the MHC region. Sixty-eight variants in the MHC II region associated with non-LS and two loci outside MHC region were identified, i.e. *PLAC9* and *ANXA11* (10q22.3) [90].

4.5 Clinical manifestations

Sarcoidosis can affect any organ, but the pulmonary system is involved in nearly all patients with sarcoidosis [76, 91]. A well-defined subgroup of sarcoidosis patients is Löfgren's syndrome (LS), discriminated by an acute onset usually fever, bilateral hilar lymphadenopathy and in some cases, parenchymal infiltrates, erythema nodosum and/or bilateral ankle arthritis or periarticular inflammation. In Scandinavia, about one-third of sarcoidosis patients are LS. Several genetic loci associated with sarcoidosis, but the HLA region is the most relevant. HLA-DRB1*0301 is strongly associated with LS and good prognosis. 95% of LS patients who are HLADRB1*03^{positive} had a resolving disease within two years, where 49%

of HLA-DRB1*03^{negative} LS patients developed a non-resolving disease. Thus, at our institution, the HLA-DRB1*0301 is used as a marker to predict remission or progression in LS patients. In the BALF of HLA-DRB1*03^{positive} patients, there is an accumulation of specific Vα2.3/Vβ22 T cell receptor-expressing $CD4^+$ T cells. The accumulation of Vα2.3/Vβ22 $CD4^+$ T cells and the expression of HLA-DRB1*03 on APCs suggest that a specific peptide is presented by HLA-DRB1*03. A vimentin peptide could be one of the offending antigens in LS. Thus, autoimmunity may contribute to the pathogenesis of sarcoidosis in these patients [82, 92-94].

The clinical picture of non-LS is heterogeneous and expand from chronic progressive organ dysfunction to asymptomatic patients with accidental chest radiographic finding. In Swedish patients with non-LS, HLA-DRB1*14, and DRB1*15 associated with the disease and they were risk factors for a non-resolving disease. In contrast, HLA-DRB1*01 and DRB1*03 protected against the disease. In particular, HLA-DRB1*03 protected against a non-resolving disease.

HLA class I alleles might also influence the disease course, and certain HLA allele-combinations was strongly associated with persistent disease (A*03, B*07, DRB1*15) [81, 94].

4.6 Granuloma formation in sarcoidosis

Generally, granulomas are defined as organized aggregates of macrophages, with functional and morphological changes, and other immune cells. Granulomas develop in response to persistent infectious and non-infectious stimuli that macrophages cannot eradicate [95]. Sarcoid granuloma consists of a central core that is surrounded by mainly $CD4^+$ T cells. The core includes macrophages and multinucleate giant cells, which are fused macrophages, surrounded by large macrophages called epithelioid cells [96].

The initial step in granuloma formation is the activation of macrophages through binding of PRRs (e.g., TLR2) to PAMPS, and/or DAMPs ligands [97, 98]. Blood monocytes of sarcoidosis patients showed higher expression of TLR2 and TLR4 as compared to healthy subjects [49]. Moreover, augmented secretion of the proinflammatory cytokines TNFα and IL-1β was observed in sarcoidosis patients after combined TLR2 and NOD2 stimulation [49]. BAL cells of sarcoidosis patients showed enhanced production of proinflammatory cytokines (TNF-α and IL-6) after stimulation of the TLR-2/1 heterodimer and decreased response after stimulation of the TLR-2/6 heterodimer [99]. BAL macrophages stimulated with SAA showed enhanced production of TNF-α, IL-18, and IL-10 in sarcoidosis patients as compared with control subjects, comprising mostly patients with chronic obstructive pulmonary disease. The effect of SAA on BAL macrophages is mediated in part

through TLR2 [100, 101]. Binding of a ligand to TLR2 initiates the activation of NF-kappa B pathway that stimulates the production of HLA-DR, co-stimulatory molecules (CD80, and CD86) and pro-inflammatory cytokines such TNF-α, chemokines, and adhesion molecules. TNF-α plays an essential role in granuloma formation and maintenance [80, 81, 102].

The next phase in granuloma formation is the interaction of MHC II-peptide complex on APCs with TCR on $CD4^+$ T cells that leads to a polarized $CD4^+$ response and the production of T-helper (T_H) cytokines such as interferon-gamma (IFN-γ) and tumor necrosis factor-α (TNF-α) [101, 103]. Furthermore, T_H17 $CD4^+$ T cells were found within granuloma of sarcoidosis patients with active or relapsing disease. Recently, T_H1/T_H17 $CD4^+$ T cells that can produce both IL-17 and IFN-γ and co-express the transcription factors T-bet and RORγT were found in BAL from LS and non-LS patients. These cells represent the majority of $CD4^+$ T cells. In non-LS, T_H17/T_H1 cells are the primary producers for IFNγ [104-106]. Furthermore, impaired regulatory T-cell function and alterations in the expression of co-inhibitory receptors that control T-cell responses, such as PD-1, CTLA-4, and BTNL2 suggest impaired T-cell regulation in pulmonary sarcoidosis. Within the granulomas, fibrosis can be induced with the production of T_H2 cytokines (IL-4 and IL-13) and TGF-β [96, 107-112].

Recent data showed that activation of mechanistic target of rapamycin complex 1 (mTORC1) in macrophages – mTORC1 senses and integrates inputs from its surrounding to regulate the metabolism and growth of many cell types, including macrophages – drives their proliferation and hypertrophy, resulting in granuloma formation. Patients with progressive sarcoidosis showed a marked increase in expression of genes in the mTORC1 pathway, suggesting that mTORC1 contributes to disease progression in sarcoidosis [110, 113].

In summary, granuloma formation in sarcoidosis is probably due to several abnormalities that engage antigen/antigens clearance, exaggerated effector T cell response, and an aberrant granuloma signaling pathway (mTORC1) in macrophages [104, 110, 114].

4.7 Diagnostic approach

The diagnosis of sarcoidosis is based on three criteria: clinical and radiographical presentation, the absence of an alternative diagnosis, and histological finding of non-caseating granuloma [115]. Histological diagnosis is not always needed and can be omitted in the setting of a classic clinical presentation, such as Löfgren's syndrome.

Chest radiographs are abnormal in 90% of cases at presentation. Lymphadenopathy occur in more than two-thirds of patients. Pulmonary infiltrates are present in 20-50% of patients. Pulmonary fibrosis occurs in 20-25% of patients [76, 115]. Pulmonary sarcoidosis is usually classified into five stages according to findings on a conventional chest radiograph (Table 1).

Table 1. Chest radiographic findings of pulmonary sarcoidosis and prognosis [107, 116, 117].

Stage	Finding	Prognosis
0	No obvious findings	Not applicable
I	Hilar or mediastinal nodal enlargement only	Spontaneous resolution occurs in 60-90%
II	Nodal enlargement and parenchymal disease	Spontaneous resolution occurs in 40-70%
III	Parenchymal disease only	Spontaneous resolution occurs in 10-20%
IV	Pulmonary fibrosis	Permanent organ dysfunction

High-resolution CT (HRCT) scan is more sensitive than chest radiograph in recognizing abnormalities and is sometimes but not regularly used for disease monitoring [76]. Pulmonary function tests (PFTs) are routinely performed and can show a restrictive or obstructive pattern. Diffusion capacity for carbon monoxide (DLCO) can be decreased.

Flexible bronchoscopy with bronchoalveolar lavage (BAL) are cornerstone investigations. During flexible bronchoscopy, endobronchial and transbronchial lung biopsy, and endobronchial ultrasound-guided transbronchial needle aspiration can also be performed. BAL is a safe and minimally invasive procedure. Bronchoalveolar lavage fluid (BALF) cells comprise several subtypes [118]. In sarcoidosis patients, increased total BALF cell counts, lymphocytosis (>15% lymphocytes) and increased CD4/CD8 ratio (>3.5) are important findings [118, 119].

4.8 Sex differenced in sarcoidosis

Sex-specific differences are known in the immune responses [120] and in different pulmonary diseases, such as asthma and idiopathic pulmonary fibrosis [121]. Therefore, we investigated the sex-specific differences in LS and non-LS sarcoidosis patients.

Comparison of LS-female versus LS-male

Compared with the LS-female group (42%), the LS-male (58%) group had higher macrophages, lymphocytes, basophils, and mast cells; lower BALF neutrophils; lower proportion of chest radiograph stage I and higher percentage of chest radiograph stage II (data not shown).

Comparison of non-LS-female vs non-LS-male

Compared with female (38%), male (62%) exhibited reduced PFTs (VC%, FVC%, TLC%). Also, male sex exhibited higher percentages of chest radiograph stage II and IV and was more predisposed to develop a non-resolving disease (data not shown). Moreover, non-resolving event associated with more likelihood with the male sex (OR: 1,48; 95% CI: 1,029; 2,12). In conclusion, male sex associated with increased disease severity as compared with the female group. The higher prevalence of male sex in this cohort is similar to prior results [122].

4.9 Age and Sarcoidosis

Another important aspect is the influence of ageing on sarcoidosis. Our results showed that the probability of non-resolving disease increase with age. A recent study showed that elderly patients (≥65 years) with sarcoidosis had more severe disease and death related to sarcoidosis was more prevalent in this group as compared to younger patients [123]. Therefore, age is a predictor of higher morbidity, i.e. non-resolving disease.

4.10 Biomarkers in sarcoidosis

Several attempts have been made to identify reliable biomarkers that could reflect the inflammatory activity in sarcoidosis. Most frequently, serological tests have been reported, e.g. the levels of serum amyloid A (SAA), beta2-microglobulin, soluble IL-2 receptor (sIL2R), and the activity of angiotensin-converting enzyme (ACE), lysozyme, or chitotriosidase. Their value in diagnosing the disease and assessing the activity has been investigated. However, they are not specific or sensitive enough to *per se* create a solid ground for decisions on how to manage the disease [124]. BALF cell characteristics could be useful biomarkers, e.g. increased lymphocytes, neutrophils, eosinophils, and mast cells [125, 126]. Currently, no single serum marker can be used for diagnosis, severity staging, or therapeutic response. However, the combination of serum and BALF differential cell counts might be useful in the appropriate clinical setting.

4.11 Treatment of pulmonary sarcoidosis

Generally, treatment is mainly based on expert opinion and best clinical practice. Many patients with pulmonary sarcoidosis are asymptomatic, and spontaneous resolution occurs in approximately 60% of patients [76]. Thus, treatment is not indicated for asymptomatic patients with only mildly abnormal lung function.

The chest physician should consider initiating treatment in the following circumstances: worsening of respiratory symptoms (e.g. dyspnea), severe pulmonary dysfunction or deterioration of lung function (>10% reduction in FVC, or >15% reduction in DLCO from baseline measurements), or radiographical progression [76]. First-line therapy is monotherapy with oral corticosteroids. Methotrexate is the first choice as second-line glucocorticoid-sparing medicine. Azathioprine is an alternative.

TNF-α is crucial for the formation and maintenance of granuloma. TNF-α release from alveolar macrophages is higher in patients who have a progressive or corticosteroid-resistance disease than in patients with stable disease. Thus, anti-TNF-α agents may be an alternative in the treatment of refractory sarcoidosis and may constitute third-line therapies after failure of glucocorticoids and/or methotrexate/azathioprine treatments [76, 127, 128].

5 AIMS

5.1 Paper I

The aim of the study was to investigate the extent of AM polarization into M1 or M2 phenotypes in LS and non-LS patients as compared to HC. Additionally, we studied the expression of the innate immune receptors TLR2 and TLR4 in AM in LS and non-LS patients as compared to HC.

5.2 Paper II

This study aimed to gain more knowledge about the expression of all three PPARs (α, β/δ and γ) in BALF AM and BALF and blood $CD4^+$ and $CD8^+$ T cells in LS and non-LS patients as compared with HC.

5.3 Paper III

The study aimed to investigate the association between sarcoidosis-associated genetic variants and quantitative levels of BALF macrophages, lymphocytes, and neutrophils in LS and non-LS, respectively.

5.4 Paper IV

The study aimed to utilize data from a BALF registry of pulmonary sarcoidosis patients (LS and non-LS) to identify BALF cells that could predict disease severity (defined as advanced chest radiographs, reduced pulmonary function, or necessity for treatment) and/or chronicity (non-resolving course after two years).

6 COMMENTS ON SUBJECTS AND METHODS

6.1 Study subjects

All included sarcoidosis patients in these studies were referred to the Lung and Allergy clinics (at Karolinska University Hospital or Södersjukhuset, Stockholm, Sweden) for an investigational work-up of clinically suspected pulmonary sarcoidosis. The diagnosis of sarcoidosis was established according to the recommendations of the World Association of Sarcoidosis and other Granulomatous Disorders (WASOG) and other expert opinions [129, 130]. The diagnosis of sarcoidosis based on three criteria: clinical and radiographical presentation, histological finding of non-caseating granuloma, and the exclusion of an alternative diagnosis. Histopathological diagnosis was not always needed and was omitted in the setting of a typical clinical presentation, such as Löfgren's syndrome [107]. BAL was performed at the time of the diagnosis, and none of the patients was on anti-inflammatory or immune-suppressive treatment at this stage. All patients had a clinical picture completely consistent with sarcoidosis, and they were subdivided into two groups according to their clinical phenotypes: Löfgren's syndrome (LS) and non-LS. Patients were also classified as being HLA allele DRB1*0301 positive ($DR3^{postive}$) or DRB1*0301 negative ($DR3^{negative}$). The percentages of the predicted percentages of vital capacity (VC), forced vital capacity (FVC), forced expiratory volume in 1 second (FEV1), total lung capacity (TLC), and diffusing capacity of the lung for carbon monoxide (DLCO) were obtained from the sarcoidosis registry. VC, FVC, FEV1, and TLC were measured in liters. DLCO was measured in mmol/min/kPa. The radiological staging based on chest radiographs according to Scadding stages (0, I, II, III, IV) [131]. Healthy individuals were non-smokers, and none of them had signs of infection or inflammation at time of BAL. Written informed consent was obtained from all subjects, and the Regional Ethical Review Board (Stockholm, Sweden) approved the studies.

6.2 Bronchoalveolar lavage (BAL) and preperation of cells

Bronchoalveolar lavage fluid (BALF) has a central role in the diagnostic workup and differential diagnosis of sarcoidosis. BALF cells comprise different cell subtypes, including macrophages, lymphocytes, and few percentages of basophils, eosinophils, neutrophils and mast cells . In conjunction with appropriate clinical and radiographic findings that are compatible with sarcoidosis, an increased total BALF cell counts, an accumulation of $CD4^+$ T cells, and consequently, an increased BALF CD4/CD8 ratio (> 3.5 %) strongly promote the diagnosis of sarcoidosis.

BAL was performed as previously described [132]. Briefly, after light sedation and topical local anesthesia, a flexible fiber-optic bronchoscope was passed trans-nasally

(in few cases trans-orally), and BAL was performed in a subsegmental bronchus in the middle-lobe using low-pressure suction technique. The expression of cell surface antigens (CD3, CD4, CD8) and the $CD4^+$ to $CD8^+$ cell ratio was performed by flow cytometric analysis using monoclonal antibodies against $CD3^+$, $CD4^+$, $CD8^+$ as previously described [133].

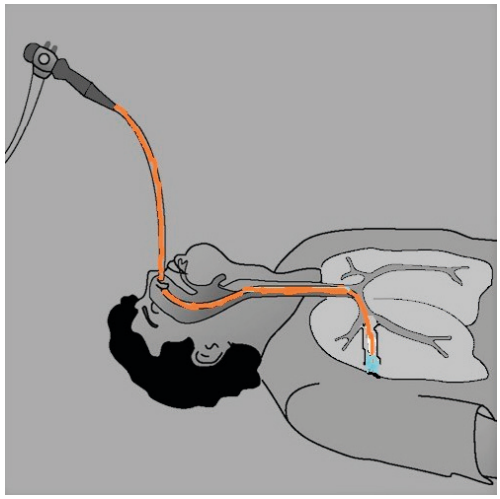

Figure 2. *Bronchoscopy of the lung simplified.*

ROC curve to determine the best cut-off value(s) of BALF CD4/CD8 ratio for the sarcoidosis diagnosis

Previous studies showed various cut-off values for CD4/CD8 ratio (ranging from 2 to 4) [134]. A previous study showed that healthy-smoker had lower CD4/CD8 ratio as compared with healthy-non-smoker [135]. Additionally, DR3 genotype (positive vs negative) may affect the CD4/CD8 ratio [136]. Thus, we determined to find the best cut-off value for entire LS patients, LS-non-smoker, LS-smoker, LS-ex-smoker, entire non-LS population, non-LS-non-smoker, non-LS-smoker, non-LS-ex-smoker, LS-$DR3^{positive}$, LS-$DR3^{negative}$, non-LS-$DR3^{positive}$, and non-LS-$DR3^{negative}$, respectively.

The most appropriate cut-off value for CD4/CD8 ratio in all groups was >3.0, and the (entire sarcoidosis patients) sensitivity, specificity, likelihood ratio (LR), and AUC was 75%, 74%, 2.8, and 0.81 respectively. Smokers had a lower CD4/CD8 ratio (but not statistically significant) as compared with non-smoker and ex-smoker. The CD4/CD8 ratio was comparable between HLA-DRB1 $*03^{positive}$ vs HLA-DRB1$*03^{negative}$ groups (slightly higher in the positive group as compared with the negative group but did not reach statistical significance) (Table 3).

Table 2. CD4/CD8 ratio cut-off values, sensitivity, specificity, likelihood ratio, and AUC. * designates the number of individuals after excluding outliers, H and P indicate healthy controls and sarcoidosis patients, respectively. N = number. AUC = area under the ROC curve. CI = confidence interval. LR = Likelihood ratio. ROC = receiver operating characteristic curve

	HN	HN*	PN	PN*	H Mean	H Median	P Mean	P Median	Cut-off	Sensitivity	95% CI	Specificity	95% CI	LR	AUC	95% CI	P-value
HC vs Sarcoidosis	38	34	1007	974	2.4	2.1	6.68	5.25	> 3.0	0.75	0.72 to 0.78	0.74	0.57 to 0.85	2.8	0.81	0.76 to 0.85	<0.001
HC vs LS	38	34	326	319			8.46	6.90	> 3.0	0.81	0.76 to 0.85	0.74	0.57 to 0.85	3.1	0.88	0.83 to 0.92	<0.001
HS vs non-LS	38	34	634	618			6.38	5.05	> 3.0	0.76	0.72 to 0.79	0.74	0.57 to 0.85	2.9	0.8	0.76 to 0.85	<0.001
Non-smoker																	
HC vs Sarcoidosis non-smoker	38	34	527	513			6.92	5.50	> 3.0	0.76	0.72 to 0.80	0.74	0.57 to 0.85	2.9	0.82	0.77 to 0.86	<0.001
HC vs LS-non-smoker	38	34	171	170			9.35	8.15	> 3.0	0.84	0.77 to 0.88	0.74	0.57 to 0.85	3.2	0.89	0.84 to 0.93	<0.001
HC vs non-LS-non-smoker	38	34	336	326			6.18	5.00	> 3.0	0.75	0.70 to 0.79	0.74	0.57 to 0.85	2.8	0.8	0.75 to 0.86	<0.001
Smoker																	
HC vs Sarcoidosis-smoker	38	34	167	156			5.43	4.30	> 3.0	0.65	0.58 to 0.72	0.74	0.57 to 0.85	2.5	0.74	0.67 to 0.81	<0.001
HC vs LS-smoker	38	34	62	60			6.90	4.95	> 3.0	0.73	0.61 to 0.83	0.74	0.57 to 0.85	2.8	0.83	0.74 to 0.91	<0.001
HC vs non-LS-smoker	38	34	92	87			5.43	4.40	> 3.0	0.68	0.57 to 0.77	0.74	0.57 to 0.85	2.6	0.75	0.67 to 0.83	<0.001
Ex-smoker																	
HC vs Sarcoidosis-ex-smoker	38	34	304	292			6.63	5.60	> 3.0	0.77	0.72 to 0.82	0.74	0.57 to 0.85	2.9	0.82	0.77 to 0.87	<0.001
HC vs LS-ex-smoker	38	34	90	87			7.97	6.70	> 3.1	0.8	0.71 to 0.87	0.74	0.57 to 0.85	3	0.89	0.83 to 0.94	<0.001
HC vs non-LS-ex-smoker	38	34	200	194			6.62	5.45	> 3.0	0.8	0.74 to 0.85	0.74	0.57 to 0.85	3	0.82	0.76 to 0.88	<0.001
HLA-DRB1*03																	
HC vs LS-HLA-DRB1*03^{positive}	38	34	201	193			8.28	6.70	> 3.0	0.78	0.72 to 0.83	0.74	0.57 to 0.85	3	0.86	0.81 to 0.91	<0.001
HC vs non-LS-HLA-DRB1*03^{positive}	38	34	87	84			6.48	4.95	> 3.0	0.77	0.67 to 0.85	0.74	0.57 to 0.85	2.9	0.82	0.74 to 0.89	<0.001
HC vs LS-HLA-DRB1*03^{negative}	38	34	99	96			7.82	6.80	> 3.0	0.85	0.77 to 0.91	0.74	0.57 to 0.85	3.2	0.9	0.84 to 0.95	<0.001
HC vs non-LS-HLA-DRB1*03^{negative}	38	34	433	417			6.58	5.00	> 3.0	0.74	0.70 to 0.78	0.74	0.57 to 0.85	2.8	0.79	0.74 to 0.84	<0.001

A meta-analysis of BALF CD4/CD8 ratio from pooled 1885 sarcoidosis patients showed that sensitivity, specificity, and AUC was 70%, 80%, 0.84, respectively. As compared with this meta-analysis, our result demonstrated slightly higher sensitivity and lower specificity. In our analysis, the control group was healthy non-smoker volunteers. In the meta-analysis, the control group(s) was healthy non-smoker individuals and other non-sarcoidosis pulmonary patients. The diversity in the control group might explain the slight difference in sensitivity, specificity. Also, our results confirm previous statements, that BALF CD4/CD8 ratio should only be used as an adjunctive tool and should be integrated with other clinical and laboratory results [107, 129, 130].

6.3 Monitoring gene expression: Real-time polymerase chain reaction (RT-PCR)

RT-PCR comprises several steps: 1- RNA isolation from specific cells; 2- reverse transcription of mRNA to cDNA; 3- amplification of specific DNA targets using polymerase chain reaction (PCR); and 4- determination of the initial concentration of the selected transcript.

6.3.1 RNA extraction and complementary DNA (cDNA) synthesis

Paper I

Total RNA was extracted via the guanidium thiocyanate-phenol-chloroform technique [137]. Briefly, cultured total BAL cells or AMs were incubated with RNA Bee (Nordic Biosite, Stockholm, Sweden). Then chloroform was added to separate the lysate into an aqueous and organic phase. Then centrifugation removes DNA and proteins from the aqueous phase that includes RNA. The RNA was precipitated from the aqueous phase by adding isopropanol. The RNA pellet was washed in 75% ice-cold ethanol, followed by air-drying. The pellet was after that dissolved in autoclaved ultra-clean water.

To synthesize cDNA, 1μg of total RNA was incubated in the presence of 20 mM random hexamers primers (Pharmacia Biotech, Uppsala, Sweden) and 200 units Superscript™II RNase H- Reverse transcriptase (Invitrogen, Lidingö, Sweden) for 10 min at room temperature and then 45 min at 40°C, followed by 5 min at 95°C to inactivate the enzyme. The cDNA samples were stored at -20°C until use.

Paper II

Depending on the cells count ($\leq 5\times10^5$ or $\geq 10^6$) total RNA was isolated using the RNAqueous-micro kit or the RNAqueous kit (Ambion®) according to the manufacturer's instructions. The quantity of total RNA was analyzed using NanoDrop

ND-1000 Spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). To synthesize cDNA, the high capacity RNA-to-cDNA kit (Invitrogen™) was used according to the manufacturer's instructions.

6.3.2 RT-PCR

The important characteristic of RT-PCR is that amplification of cDNA is detected in real-time as PCR is progressing by using a fluorescent reporter. The fluorescent reporter signal intensity is directly proportional to the number of amplified DNA molecules. For relative quantification of the expression of cytokine genes in BALF cells, the following 2-ΔΔCT method was used [138]. In the first step, the Ct of the target gene (e.g. TLR2) was normalized to the endogenous control (β-actin: paper I, Proteasome Subunit, Beta Type, 2 (PSMB2): paper II). In the following step, normalization to the mean value of the target gene expression (e.g. TLR2) in a healthy control group (calibrator sample) was performed. The samples were run in duplicates to assure the validity of the experimentation.

Paper I

Real-time PCR was used to quantify the relative gene expression of M1 associated markers (IL-12p35, IL-12p40, IL-23p19, CCR7, iNOS, CXCL10, CXCL11, CXCL16, CCL20, CD80, CD86), M2 associated markers (IL-10, CCR2, CCL18), innate immune receptors (TLR2, TLR4, TLR9), and IL-7A, using ABI Prism 7700 Sequence Detection System (Applied Biosystems, Foster City, CA, USA).

Paper II

Real-time PCR was used to quantify the relative gene expression of PPAR α, β/δ, γ in BALF cells.

6.4 Flow cytometry

The immunologist uses flow cytometry for multiple purposes, e.g. to immunophenotype cells, measure intracellular cytokines and transcription factors, asses viability, or sort cells. Flow cytometry enables the researcher to obtain specific information about individual cells in a mixture of various cell populations.

A monoclonal antibody that is attached to a fluorochrome (e.g. FITC anti $CD4^{+}$) is used to detect (stain) a specific extra- or intracellular protein (antigen). In a flow cytometer, cells are required to flow past the laser light one cell at a time. A flow cytometer detects fluorescence and light scattered from every single cell. A detector in front of the light beam measures forward scatter, and multiple sensors to the side of the light beam sense side scatter. The path of light scattered by the cell corresponds to cell size and granularity. The fluorescent light emitted from stained sells is detected by sensors that can recognize emitted photons with a specific wavelength.

Paper II

Flow cytometry was used to detect intracellular PPAR α, β/δ, γ on a single cell level in blood and BALF cells. Cells were analyzed on BD FACSCanto II flow cytometer (BD Bioscience), and data were processed using the web-based application Cytobank [139] (Cytobank, Inc).

Flow cytometric sorting of BAL macrophages and $CD4^+$ T cells

BAL cells were stained with PE Mouse Anti-human CD4, clone RPA-T4. The stained cells were sorted by FACSVantage (BD Biosciences). BAL cells were gated on lymphocytes and macrophages, and the stained $CD4^+$ T cells and macrophages were effectively separated. The purity of the sorted population, which was determined by flow cytometry, was approximately 98%.

6.5 Statistical analysis

We performed the nonparametric Mann-Whitney test and Kruskal-Wallis test to compare continuous variables. In the case of the Kruskal-Wallis test, Dunn's post-test was used to compare the difference between groups. Fisher's test and chi-square test were used to compare categorical variables. Wilcoxon matched-pairs signed-rank test was used to compare two matched samples. Spearman correlation coefficient was calculated for the correlation between variables. P-values of <0,05 were regarded as significant. We used the software GraphPad Prism for Windows (GraphPad Software, San Diego, California USA) to perform the analysis.

The receiver operating characteristic (ROC) curve was used to determine a proper cut-off value of CD4/CD8 ratio. Furthermore, we determined the most appropriate cut off-value for CD4/CD8 ratio in the entire sarcoidosis patients, and in sarcoidosis subgroups: total LS patients, LS-non-smoker, LS-smoker, LS-ex-smoker, entire non-LS population, non-LS-non-smoker, non-LS-smoker, non-LS-ex-smoker, LS-HLA-DRB1*03^{positive}, LS-HLA-DRB1*03^{negative}, non-LS-HLA-DRB1*03^{positive}, and non-LS-HLA-DRB1*03^{negative}. The most appropriate cut-off value was selected as that which combined maximal sensitivity with the best specificity. We used the ROUT method to exclude outliers in continuous variables (Q = 0,1%). Then, we performed the Grubb's test for outlier testing to validate that the p-value was >0,05. We used the software GraphPad Prism version 8.2.1 for Windows (GraphPad Software, San Diego, California USA), and Minitab version 19.1.1 (Minitab 19 Statistical Software (2010). State College, PA: Minitab, Inc.) to perform the tests.

Binary logistic regression method was used to predict the impact of the independent variable(s) (e.g. mast cells ≤10, mast cells >10) on the dependent binary variable (e.g. resolving vs chronic). Minitab version 19.1.1. to perform the analyses and GraphPad Prism version 8.2.1 for Windows (GraphPad Software, San Diego, California USA) were used to perform the analysis.

7 RESULTS AND DISCUSSION

This section will introduce the main findings of each paper, followed by a brief discussion.

7.1 Paper I

No evidence of altered alveolar macrophage polarization, but reduced expression of TLR2, in bronchoalveolar lavage cells in sarcoidosis

Main results and discussion

We found that AM of healthy individuals express both M1 and M2 markers in steady state. Mitis et al., found the same result [20].

In freshly isolated AMs, the relative gene expression of CCL18 (an M2 associated marker) was significantly higher in sarcoidosis patients compared to healthy individuals. Several studies found a positive correlation between BALF CCL18 level and fibrotic lung diseases, e.g. idiopathic lung fibrosis, stage III and IV hypersensitivity pneumonitis, and systemic sclerosis [140-142]. Also, plasma level of CCL18 was suggested to be a marker of disease activity [124, 143]. In our study, no correlations between CCL18 mRNA expression and chest radiograph stages or lung function parameters were found. But, most of our patients had chest radiograph stage I or II and only one patient had a chest radiograph of stage IV. Our finding is in concordance with the study by Cai et al., and again in this study, most of the patients had chest radiographic stage I and II, and only four patients had stage IV [144]. We did not observe any difference in CCL18 expression between LS and non-LS patients. Accordingly, CCL18 might serve as a T cell chemoattractant, in the early stages of the disease, and might exhibit a profibrotic role in more advanced disease. Interestingly, in mouse lungs CCL18 induced long-term pulmonary infiltration of T lymphocytes and consequently infiltration-dependent deposition of collagen through a TGF-β1-dependent mechanism [145].

Compared with healthy individuals, we found that AMs of sarcoidosis patients exhibited lower gene expression of TLR2. This finding was in disagreement with our previous published study of higher expression of TLR2 and TLR4 receptors on blood monocytes [49]. However, it should be emphasized that different cell types were analyzed (monocytes vs macrophages) and different methods were used (cell surface receptor expression vs mRNA expression). One reason for this discrepancy in TLR expression between lung and blood could be variations in exposure to different stimuli known to influence TLR expression. For instance, TLR ligands can up- or downregulate TLR mRNA expression depending on dose and time of the stimulus [49, 146, 147]. Moreover, the cell surface expression

of TLR2 was found to be increased by some cytokines and decreased by others [148], e.g. IFNγ and TNF downregulated TLR2 expression on human monocytes; although macrophages were not studied. Hence, the variety of inflammatory mediators presented in different compartments might contribute to the local TLR expression. Furthermore, the TLR2 down-regulation was mainly observed in LS patients, and this might indicate that AM encountered a specific microbial ligand that specifically binds to TLR2. Additionally, downregulation of TLR2 might have a role in the resolving of granuloma in LS patients.

In conclusion, the reduced gene expression of TLR2 (M1 associated marker) and the increased expression of CCL18 (M2 associated marker) in AM of sarcoidosis patients could indicate a shift toward M2-like macrophages. Recently, Shamaei *et al.*, found enhanced $CD163^+$ (M2 associated marker) staining of tissue samples (lung and lymph node) from sarcoidosis patients as compared with tuberculosis patients [149]. Furthermore, Locke *at al.*, found that treatment of peripheral blood mononuclear cells (PBMCs) from sarcoidosis patients with purified protein derivative (PPD)-coated polystyrene beads resulted in the formation of granuloma-like multicellular aggregates and alternative (M2) macrophage polarization [150, 151].

7.2 Paper II

Reduced expression of peroxisome proliferator-activated receptor-alpha in BAL and blood T cells of non-Löfgren's sarcoidosis patients

Main results and discussion

The expression of PPARs mRNA was investigated in total BALF cells in sarcoidosis patients and HC. In sarcoidosis patients, the mRNA expression of PPARα, PPARβ/δ and PPARγ did not differ significantly from HC. In non-LS patients, PPARβ/δ mRNA was increased as compared with LS patients and HC.

Compared with HC, LS and non-LS patients exhibited lower PPARα mRNA expression in FACS-sorted $CD4^+$ T cells. Nevertheless, the mRNA expression of PPARα, PPARδ and PPARγ in FACS-sorted AM did not significantly differ in HC, LS and non-LS.

Furthermore, BALF $CD4^+$ and $CD8^+$ T cells of non-LS patients showed lower PPARα level as compared with LS patients. Additionally, PPARα and PPARγ levels were reduced in blood $CD4^+$ and $CD8^+$ T cells of non-LS patients as compared with LS. In AM, PPARs levels were comparable in LS, non-LS and HC.

Several studies showed that PPARα and PPARγ expression were downregulated in various inflammatory disorders. Furthermore, PPARγ expression was reduced

in PBMCs of multiple sclerosis patients and the colon of ulcerative colitis patients [152, 153]. Also, PPARγ expression was reduced in lung tissue and epithelial cells of COPD patients [154].

Idali *et al*., showed that the mRNA expressions of IFNγ and TNFα in BALF cells were reduced in $DR3^{pos}$ sarcoidosis patients (typically with Löfgren's syndrome and resolving disease course) as compared with $DR3^{neg}$ patients [155]. Therefore, the reduced expression of PPARα in BALF and blood $CD4^+$ and $CD8^+$ T cells could contribute to higher expression of IFNγ and TNFα and in non-LS patients that are typically $DR3^{neg}$ and prone to develop a non-resolving disease course. Interestingly, studies showed that PPARα or PPARγ ligand activation decreases the expression of inflammatory cytokines, such as IFNγ and TNFα by T_H1 cells [156, 157]. Also, Fenofibrate (PPARα agonist) reduced the inflammatory response in allergen-induced airway inflammation in mice [158, 159]. Rosiglitazone (PPARγ agonist) treatment of patients with active ulcerative colitis or smokers with mild to moderate asthma resulted in clinical improvement [160, 161]. Thus, the reduced expression of PPARα or γ in $CD4^+$ and $CD8^+$ T cells in non-LS patients could act as a potential marker for disease activity and that treatment with PPARα and γ agonists might have a role in the treatment of non-LS patients with active or progressive disease.

In conclusion, we demonstrated for the first time, that PPARα and PPARγ expressions are downregulated $CD4^+$ and $CD8^+$ T cells in non-LS patients as compared to LS patients.

7.3 Paper III

Influence of sarcoidosis risk genetic variants on the quantitative level of bronchoalveolar lavage fluid (BALF) cell populations

Main results and discussion

Our results show that genetic variants associated with LS and located in the extended MHC region [90, 162] associated with the quantitative levels of BALF macrophages, lymphocytes, and neutrophils. Also, genetic variants associated with non-LS and located in the MHC class II region [90] were associated with the quantitative levels of BALF macrophages. These findings indicate a functional relationship between sarcoidosis genetic susceptibility and quantitative levels of BALF cells, which may modulate immune system functions. Interestingly, several LS and non-LS genetic variants that associated with BALF cell types exhibit regulatory effect in lung, blood, T cells, B cells, macrophages, and neutrophils.

Few limitations of this study can be addressed. Firstly, this study used only the SNPs that were associated with LS and non-LS, respectively. These SNPs represent only minority of the SNPs included in the Immunochip. Therefore, the association of other SNPs on other chromosomes that could also affect the quantitative level of BAL cell populations, was not evaluated. Furthermore, the genetic variants that associated with the quantitative levels of BALF cells are located within the MHC region are in strong linkage disequilibrium, and this should be considered when interpreting the results. Third, no association between genetic variants and BALF counts of basophils and eosinophils (in LS and non-LS) could be analyzed because a lot of the counts are nil.

In summary, our finding indicates that genetic variants associated with LS and non-LS have a pleiotropic effect on the quantitative level of BAL cell subpopulations and this effect can contribute to the observed differences in BALF cell counts between LS and non-LS patients.

7.4 Paper IV

Bronchoalveolar lavage fluid cell subsets associate with the disease course in Löfgren's and non-Löfgren's sarcoidosis patients

Main results and discussion

This study illuminates the prognostic utility of BALF cells in newly diagnosed sarcoidosis patients. Compared with LS-resolving, LS-chronic patients exhibited higher percentages of BALF lymphocytes, neutrophils, and eosinophils. Also, in newly diagnosed LS patients, increased BALF lymphocytes (>24 x10^6/L) was more likely to predict a chronic disease course. In LS patients, DRB3^{pos} is a marker for the best prognosis, i.e. resolving disease [163, 164] but HLA-genotyping might not be widely applied. Accordingly, increased BALF lymphocytes might be used as an alternative or complimentary predictor of chronicity when combined with other proper clinical parameters, e.g. older age at disease onset, and more advanced chest radiograph stages. Moreover, elevated BALF neutrophils and basophils associated with more severe disease, i.e. reduced TLC.

In sarcoidosis patients, DR3^{pos} and DR15^{pos} are markers for a resolving disease (best prognosis) and a chronic and progressive disease course (poor prognosis), respectively [165, 166]. BALF percentages of lymphocytes, eosinophils, basophils, and mast cells count were higher in non-LS-DR15^{pos} patients as compared with LS-DR3^{pos} patients. Also, BALF mast cells were higher in non-LS-chronic patients as compared with non-LS-resolving patients. Additionally, higher BALF mast cells associated with more severe disease in newly diagnosed non-LS patients. Moreover, increased BALF basophils predicted the likelihood of treatment in newly diagnosed non-LS patients.

Our results confirm previously published findings in smaller studies that increased BALF mast cells, neutrophils, and eosinophils associate with a more severe disease course in non-LS patients, i.e. the majority of sarcoidosis patients [126, 167-169].

Neutrophils, eosinophils, basophils, and mast cells play a vital role in innate and adaptive immunity [26, 38, 39, 170-173]. For instance, mast cells, eosinophils, basophils, and neutrophils can induce T cell activation, proliferation, and cytokine secretion in an antigen-dependent manner [28, 38, 41, 172]. Mast cells can decrease the suppressive activity of Tregs [174] and can promote T_H1 and T_H17 responses [42, 43]. These data suggest that BALF mast cells, neutrophils, eosinophils, and basophils might contribute to the inflammatory response, granuloma formation and maintenance, disease severity and progression in pulmonary sarcoidosis.

In conclusion, this study suggests that increased BALF percentages of neutrophils, eosinophils, lymphocytes, basophils and notably mast cells might be used as prognostic markers in newly diagnosed sarcoidosis patients.

8 CONCLUDING REMARKS

- In freshly isolated alveolar macrophages (AM) of sarcoidosis patients, we identified a reduced gene expression of TLR2 (M1 associated marker), especially in patients with LS. In addition, there was an increased expression of the M2 associated marker CCL18 in sarcoidosis versus controls (HC), indicating a shift toward M2-like macrophages.
- We found no significant difference in PPARs expression in AMs of sarcoidosis patients and HC.
- In non-LS patients, PPARα expression was downregulated in BALF and blood $CD4^+$ and $CD8^+$ T cells, and the expression of PPARγ was reduced in blood $CD4^+$ and $CD8^+$ T cells.
- PPARα and CCL18 could potentially be used as biomarkers of active or advanced disease.
- Genetic variants associated with LS are significantly associated with the quantitative levels of BALF macrophages, lymphocytes, and neutrophils.
- Genetic variants associated with non-LS are associated with the number of AM.
- Several sarcoidosis risk gene variants -that are also expressed in lung and immune cell types- control the expression of nearby genes, thus highlighting a pleiotropic phenomenon between disease genetic susceptibility and the local immune response (the lungs).
- BALF cell subsets have prognostic implications in sarcoidosis.
- As compared to LS-$DR3^{pos}$ patients, non-LS $DR15^{pos}$ patients exhibited higher BALF lymphocytes, eosinophils, basophils, and mast cells.
- Overall, signs of an exaggerated immune response, reflected by lung accumulations of different cell-types, usually associated with a more severe and chronic disease.

9 FUTURE PERSPECTIVES

In the era of precision and individualized medicine, the clinician needs to consider multiple clinical parameters, biomarkers, the genetic variability of the disease, and different disease endotypes or deep phenotypes to predict the prognosis and or disease activity and to choose the appropriate treatment. One example is CCL18, which potentially could be used as a biomarker to predict patients at higher risk of progressing to lung fibrosis, another is PPARα, which could have prognostic and therapeutic implications, though further studies are required.

Non-LS patients constitute a heterogeneous group and geno- and endotyping (signifying subtypes of the disease) this group more in detail is of clinical relevance. We have shown that subtyping this group of mixed patients according to BALF cell characteristics (lymphocytic, neutrophilic, basophilic, eosinophils, and increased mast cells count) could help in predicting the disease course. Cytospin with specific cell staining is the procedure currently used, however time-consuming, to determine the BALF cell counts. Flow cytometry could alternatively be used to determine the accurate counts of different BALF cells quickly and correctly, and to evaluate the expression of various activation- and cell line specific-markers. We have shown that some of our previously identified sarcoidosis associated genetic variants can influence the BALF cell number. In the future, more detailed genome-wide association studies applied on more significant numbers of clinically well-characterized patients may reveal new genetic variants that could influence differential BALF cells and the inflammatory process in sarcoidosis.

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